Acta Biologica Szeged *18* (1—4), pp. 71—80 (1972)

LIGHT-INDUCED TRANSFORMATIONS OF PIGMENTS I. TRANSFORMATIONS OF CAROTENOIDS UNDER AEROBIC AND ANAEROBIC CONDITIONS

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(Received January 15, 1972)

Introduction

The carotenoids in chloroplast undergo continual transformation, and are in dynamic equilibrium. Carotenes and xanthophylls occur in great quantities in the photosynthetically active tissues. Several views exist as to the role of yellow pigments in the plast:

a) The carotenoids function as primary light receptors and light transformers, and they are also responsible for the absorption of light in the ultraviolet, blue and green regions (RABINOWITCH, 1969). In the case of green algae their energy-transmitting role is outstanding (EMERSON—LEWIS, 1943, ARNOLD—OPPENHEIMER, 1950). The energy of excitation of the carotenoids is taken over by chlorophyll b and transferred to the chlorophyll a molecule (FRANCZKOWIAK—SALAMON, 1970). The energy absorbed by the carotenes may also be immediately passed on to the chlorophyll a molecule (GOEDHEER, 1969).

b) As protective pigments they absorb the harmful light-rays in this way protecting the chlorophylls, proteins and other porphyrins from photodestruction. The harmful effect of light-rays is revealed primarily in the presence of O_2 (ANDERSON, 1958; ANDERSON—ROBERTSON, 1961). The light-filtering role of carotenoids is particularly important in the etiolated leaves turning green (BOGORAD, 1965).

c) The carotenoids play a considerable organizing part in the formation of the thylakoid membranes and multi-enzyme complexes. In the event of a deficiency of carotenoids, the characteristic organization of the membranes and pigments within the chloroplast either changes or does not come about at all (FALUDI—DÁNIEL et al., 1966; GYURJÁN et al., 1969; NAGY et al., 1967).

d) In the transformations of carotenoids their metabolic importance is also revealed. Xanthophyll transformations take part to a considerable extent in the development of photosynthetic oxygen (DONOHUE et al., 1967; SAPOZHNIKOV, 1969). The epoxidation deactivates the excited chlorophyll-oxygen complexes, in this way providing protection against the photosensitized oxidation of chlorophyll that would otherwise be fatal for the cell (KRINSKY, 1966).

The transformations of carotenoids depend to a large extent upon the light conditions, the temperature, the water content and the composition of the gases in contact with the plant. For example, according to CLAES, (1967) the most important transformations of the carotene precursors accumulated in the dark in a mutant of *Chlorella vulgaris* are as follows:

Phytoene
Phytofluene
ζ-Carotene $\xrightarrow[N_2]{light}$ Proneurosporene
Prolycopene

α-Carotene
β-Carotene
β-Zeacarotene

β-Carotene $\xrightarrow[O_2]{dark}$ Xanthophylls

Under anaerobic conditions light induces the cyclization of acyclic carotenes. A further transformation takes place in the presence of oxygen even in the dark. These reactions are hydroxylation and epoxidation that lead to the formation of xanthophylls.

Epoxidation and desepoxidation are the most important of the transformation reactions of xanthophylls. According to DONOHUE et al. (1967) the transformation takes place via the following intermediates:

$$\text{Zeaxanthin} \underset{light}{\overset{dark + O_2}{\rightleftharpoons}} \text{Antheraxanthin} \underset{light}{\overset{dark + O_2}{\rightleftharpoons}} \left.\begin{matrix} \\ \\ \end{matrix}\right\} \text{Violaxanthin}$$

$$\text{Lutein} \underset{light}{\overset{dark + O_2}{\rightleftharpoons}} \text{Neoxanthin} \underset{light}{\overset{dark + O_2}{\rightleftharpoons}} \left.\begin{matrix} \\ \\ \end{matrix}\right\}$$

The role of light and the gases in contact with plant in the reaction is well demonstrated by the transformations. It is not known from investigations to date whether the reaction carotene ⟶ xanthophyll can be reversed in the plasts formed, and if so, under what conditions and to what extent.

The increase of carotenes in the leaves of plants, possibly at the expense of xanthophylls, is of direct practical importance. The transformation of carotenes to vitamin A in both animal and human organisms is well known.

The inhibitory effect of oxygen on the fixation of CO_2 during photosynthesis has been known as the Warburg effect since 1920 (GIBBS et al., 1967). Our assumption that the caroteniod transformations are closely related with photosynthesis leads to the conclusion that the change of carotenoids must be influenced by CO_2. According to SAPOZHNIKOV (1969), in the event of a complete lack of CO_2 in the leaves the epoxidation is inhibited. It is not known, however, what direct effect is exerted by the carbon dioxide on the other carotenoid transformations listed above. The problems outlined have many aspects, but in the course of our investigations we wanted primarily to find answers to only the following questions:

1. During short experiments, how does strong light (40—60,000 Lux) under aerobic and anaerobic conditions effect the carotenoid content of isolated leaves and the change of this.
2. Can the transformation carotene ⟶ xanthophyll be reversed, and are lutein and zeaxanthin dehydroxylated on the action of light.
3. What is the effect of CO_2 upon the desepoxidation reactions.

Materials and Methods

Isolated leaves were used from the plants *Nonea lutea* (DESV) DC; *Raphanus sativus* L. var. *niger* (MILL) *DC.* f. *vulgaris* DC and *Spinacia oleracea* L, all grown in the field. The completely developed, but not old leaves were collected at 6 a. m. Disks of 1 cm^3 diameter were prepared from the leaves with a cork-borer by the „leaf-half" method. Four to five treatments were carried out simultaneously in parallel:

a) control,
b) illuminated in air,
c) illuminated in N_2,
d) illuminated in $N_2 + CO_2$,
e) illuminated in O_2.

Sixteen disks were used for each experiment. 5×16 leaf-disks were taken from sixteen leaves, great attention being paid to the homogeneous distribution of the leaf-disks in the parallel experiments. For example, in the case of illumination in N_2 the sixteen leaf-disks were from the middle parts of sixteen leaves but from sites of different positions. The fresh weight of the sixteen leaf-disks was 0,340 g, the dry weight 0,045 g, and the surface 12,56 cm^2. The illumination of the leaf-disks was carried out in a glass vessel of 6,8 cm diameter (of our own design), covered with a colourless polythylene foil. During the illumination, the leaf-disks lay upper surface upwards in the vessel on a plastic net, and were in a humid environment.

For lighting two 1000 W iodine-vapour lamps (Tungsram Halogen) were used that gave together a light of 60,000 Lux even after water filtration. The extraction and separation of the pigments were performed by the method of MARÓTI and GABNAI (1971). After illumination, the leaf-disks were ground to pieces in the presence of a little $MgCO_3$ in a mortar cooled with 1 ml cooled absolute acetone and about 8 ml petroleum ether (b. p. 80 °C). The above solvent mixture was divided into four parts and the plant material was „washed" four times, so that only the total pigment extract was decanted. When the pigment extraction was complete, the extract volume was adjusted to 5 ml. The total pigment extract thus obtained contains all of the carotenes, xanthophylls and clorophylls practically unchanged. The extract can be used immediately for thin-layer chromatographic separations.

The chlorophylls, carotenes and xanthophylls were separated by, thin-layer chromatography (MARÓTI and GABNAI, 1971). For preparing the layer, 10 g cellulose powder (MN 300) and 10 g silica gel (G to Stahl) powder were mixed for 1 minute with 78 ml distilled water (with an electric mixer) into a homogeneous pulp 8 ml of the pulp was transferred by means of a pipette to an 18×5 cm degreased and dry glass plate. Spreading was performed with a glass--rod, the layer thickness being 200—300 μ. The plates were left to dry in a horizontal dust--free place for 8 to 12 hours and then activated at 120 °C for 2 hours. The plates may be used immediately after cooling. The prepared plates are best stored in a desiccator.

1 ml of the petroleum ehter pigment extract was placed in line on a plate. The starting point was three cm from the plate edge. If two plates were coated with the material simultaneously, as a result of the rapid evaporation of the petroleum ether the work was contonuous. In the development of the chromatogram the spots need not be dried.

The chromatogram is developed in the dark in a refrigerator. With the following mixture: benzene-petroleum-ether-absolute ethanol-isobutanol-acetone-water = 25 : 25 : 4 : 1 : 2 : 0,4. A one--dimensonal ascending method was applied. The distance of development was 10—14 cm. The running time was about 30 min.

For the spectrophotometric investigations the pigment zones were peeled off wet lest the epoxide carotenoids undergo change. A razor blade is the most suitable for detaching them. The single pigments were eluted in methanol and centrifuged, and the absorption curves of the pure materials so obtained were recorded with a UNICAM SP 800 spectrophotometer. The quantitative changes in the carotenoids were measured with a MOM 201 spectrophotometer.

Results and Duscussion

Transformation of carotenoids

In the course of our investigations it was found that the carotenoids undergo continual transformation in the chloroplast both in the light and in the dark. The transformation of the xanthophylls are the most considerable. Carotenoid transformations are primarily light-induced reactions, but they also

depend upon the composition of the gas atmosphere around the plants, the temperature, and the water content. The most important forms of transformations are:

a) cyclization of the acyclic carotene precursors,
b) hydroxylation and dehydroxylation,
c) epoxidation and desepoxidation reactions.

The transformations of carotenoids were classified by DONOHUE et al., (1967) on the basis of the literature data and their own experimental results. We too observed the succession of transformation reactions suggested by them, and the Table has been supllemented by our own results (Fig. 1). In contrast with the literature data, it was found that on the action of light zeaxanthin and lutein are dehydroxylated and hence carotenes are formed. Light-induced reactions under aerobic conditions.

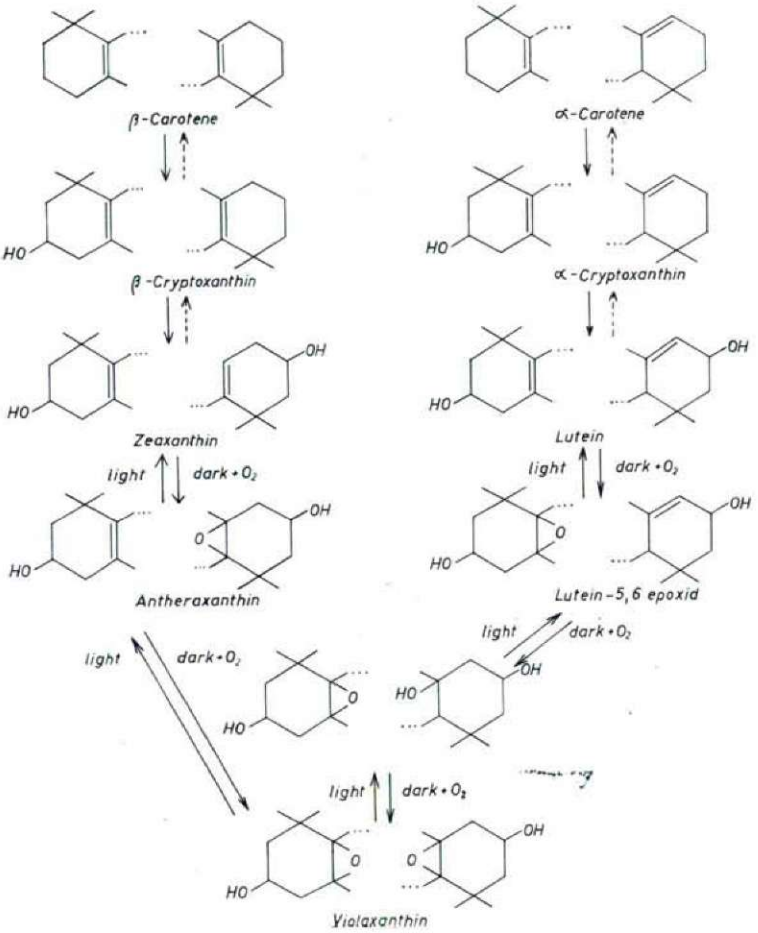

Fig. 1. Transformations of carotenoids.
Modified on the basis of DONOHUE et al. (1967). The dashed arrows the directions of the reactions assumed by us.

Table I. Percentage distribution and change in ratio of the carotenoid content of a leaf of *Nonea lutea*, as a result of illumination under different conditions (60 000 Lux, 15 minutes; the temperature below the leaf das 24 ± 2 °).

	$\beta + \alpha$ carotene	zeaxanthin lutein antheraxanthin	violaxanthin	neoxanthin	total carotenoids
control, dark	22,7	37,4	25,6	14,3	100,0
control, illuminated	25,5	50,5	9,0	14,9	106,3
O_2	24,2	48,9	12,4	14,5	108,0
N_2	26,4	52,5	7,5	13,6	111,5
$N_2 + CO_2$ (0,81%)	25,3	48,3	12,7	13,7	101,0

Two parallel series of 5 experiments were carried out under aerobic conditions: in air and in pure oxygen. Strong illumination for 5—40 minutes significantly transformed the $\beta + \alpha$ carotene, the zeaxanthin + lutein + antheraxanthin, the neoxanthin and the violaxanthin contents of the isolated leaf.

The following transformations were induced in the carotenoid content of the *Nonea lutea* leaf by illumination for 15 minutes:

a) The total carotenoid content increased by 6% in the air, and by 8% in O_2 (Table I).

b) The $\beta + \alpha$ carotene content increased by 15—19%.

c) On illumination, the greatest change was shown by the amount of lutein + zeaxanthin + antheraxanthin: it increased by 43% in the air, and 41% in oxygen.

d) The amount of neoxanthin increased by 9—10%.

e) As compared to the control, the amount of violaxanthin in the illuminated leaf decreased by 62% in the air, and 47% in oxygen.

In the case of *Raphanus sativus*, an entirely similar tendency was found in the changes in the carotenoids. The results are shown in a stereo-graph (Fig. 2).

Table II. Change in the carotenoid content of a leaf of *Nonea lutea* as a result of a 60,000 Lux illumination for 15 minutes.

	$\beta + \alpha$ carotene %	zeaxanthin %	neoxanthin %	violaxanthin %
control, dark	100	100	100	100
control, illuminated	119,4	143,7	110,4	37,6
O_2	115,3	141,2	109,1	52,4
N_2	129,9	156,6	105,6	32,5
$N_2 + CO_2$ (0,81%)	112,6	130,6	96,5	50,2

Light-induced reactions under anaerobic conditions

Under anaerobic conditions two series of 5 experiments were carried out: in pure N_2 and in N_2 containing 0,81% CO_2. In the absence of oxygen, the epoxidation is inhibited. If previously illuminated leaves are placed into nitrogen, then according to SAPOZHNIKOV (1969) the violaxanthin content does not

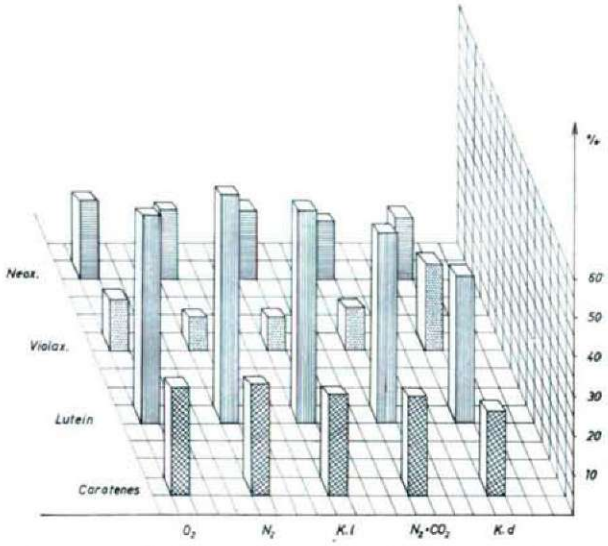

Fig. 2. Light-induced carotenoid changes in a leaf of *Raphanus sativus* under aerobic and anaerobic conditions.
The illumination lasted for 20 minutes; O_2 = in oxygen, N_2 = in nitrogen, K. 1 = in a normal atmosphere, $N_2 + CO_2$ = in nitrogen containing 0,81% CO_2, K. d. =dark control.

change. This inhibition, however, is reversible because if the leaves are replaced into the air the epoxidation again takes place in the dark.

The carotenoid transformation is increased as a result of illumination under anaerobic conditions. In N_2 the rates of the desepoxidation reactions are increased by anaerobiosis.

After illumination for 15 minutes under anaerobic conditions, the following changes may be observed in the carotenoid content of a leaf of *Nonea lutea:*

a) The total carotenoid content is the highest in N_2. It has increased by 11,5% as compared to the control (Table I). The N_2 gas containing 0,81% CO_2 inhibits the increase of the amount of total caratenoid as compared to the illuminated control.

b) In N_2 gas, as compared to the control, the amount of $\beta + \alpha$ carotene has increased by 29% and is the highest relative to the other experiments (Table II).

c) The total amount of zeaxanthin + lutein + antheraxanthin is the highest in N_2, having increased by 56% compared to the control.

d) Under anaerobic conditions, the violaxanthin is the least; that is it has undergone the greatest transformation, the decrease being 68%.

e) The light-induced desepoxidation and dehydroxylation in the leaves are inhibited in $N_2 + CO_2$. As compared with the illuminations in N_2, O_2 and air, the percentage increases of the amounts of carotene and zeaxanthin + lutein + antheraxanthin are the least here. The decrease of neoxanthin, which was not observed in the other experiments, is stricking (Table II).

The effect of a longer illumination (for 20 to 40 minutes) on the changes in the carotenoids of the spinach leaf was investigated in nitrogen and oxygen. The transformation tendency is similar to those reported above.

The characteristics established by SAPOZHNIKOV (1969) for the violaxanthin cycle were also observed by us. Apart from the epoxidation and desepoxidation reactions it was also found that hydroxylation and dehydroxylation play an important role in the transformation of carotenoids. The sensitivities of the epoxidation and desoxidation reactions are different under various experimental conditions. For instance, if the leaves are under aerobic conditions in a completely carbon dioxide-free environment, the epoxidation is inhibited even in the dark. At the same time, the light-induced desepoxidation is decreased by CO_2

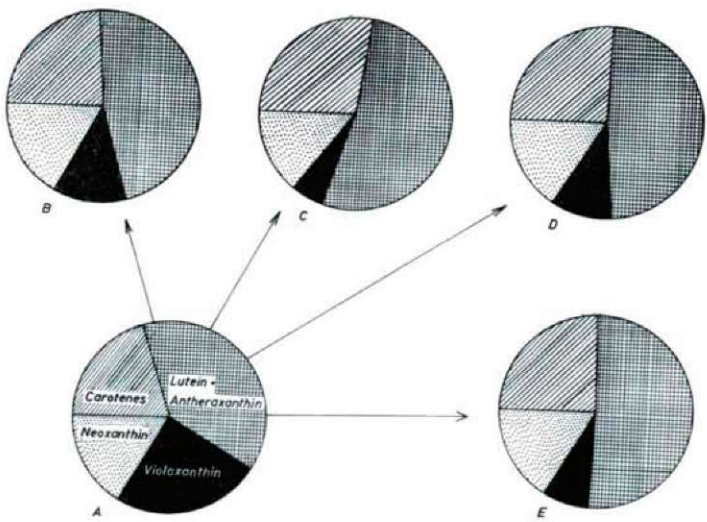

Fig. 3. Light-induced carotenoid changes in a leaf of *Nonea lutea* under aerobic and anaerobic conditions.
A = dark control; illumination for 20 minutes; E = in oxygen.
B = in a normal atmosphere; D = in nitrogen containing 0,81% CO_2;
C = in nitrogen;

in any concentration under either aerobic or anaerobic conditions. However, the extents of the two inhibitions are different. The increased sensitivity of the epoxidation reactions is shown too by their dependence upon temperature. The desepoxidation reactions stop only at —10 °C, whereas the epoxidation reactions are very slow even at +15 °C, and are completely inhibited at +2 °C (SAPOZHNIKOV, 1969).

Epoxidation reactions of xanthophylls

It may be seen from the preceding data that desepoxidation takes place under both aerobic anaerobic conditions. To try to achieve a record epoxidation, the leaf-disks were first illuminated in a normal atmosphere, in humid air for 20 minutes. The desepoxidation was then stopped, and the

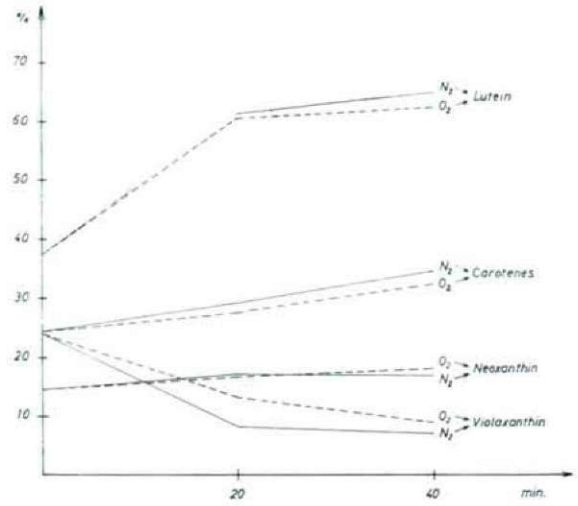

Fig. 4. Light-induced changes in the carotenoids of a leaf of *Spinacia oleracea* in N_2 and O_2.

leaf-slices were put into the dark. For the joint study of desepoxidation and epoxidation (as described above) 4×16 disks were cut from 16 leaves. With these the following four parallel experiments were performed:

1. The pigments were extracted from the control leaf-disks, without illumination.

2. After an illumination of 20 minutes, the carotenoids and chlorophylls were immediately extracted.

3. The leaf-slices were kept in a normal atmosphere in the dark fo three hours after being illuminated and only then were extracted the leaf-pigments.

4. The leaf-disks were placed after illumination into a N_2 atmospher efor three hours, and the pigments then extracted.

The experiments with the leaf of Nonea lutea were repeated four times. The avarage results so obtained in this way were tabulated (Table III).

Table III. Carotenoid changes coused by dark- and light-induced reactions in a leaf of *Nonea lutea.*

	carotenes %	zea-xanthin lutein %	anthera-xanthin %	neo-xanthin %	viola-xanthin %	total caroten-oids %
1. control, dark	22,8	33,6	3,7	15,1	24,8	100,0
2. control, illuminated	28,0	47,2	8,7	14,9	10,7	109,5
3. kept for 3 hours in the dark in air	24,8	43,3	5,1	15,9	22,6	111,8
4. kept for 3 hours in the dark in N_2	23,6	39,4	6,2	13,7	18,1	100,9

From the results it can be seen that:

a) As a result of desepoxidation and dehydroxylation on illumination, the amounts of carotenes, and lutein + zeaxanthin + antheraxanthin increase, while that of violaxanthin decreases.

b) As a result of epoxidation and hydroxylation in leaf-slices previously illuminated and then kept in the dark, the amount of violaxanthin increases and that of carotenes decreases. The increase of the amount of violaxanthin is due to the reaction (DONOHUE et al., 1967):

zeaxanthin
lutein
antheraxanthin $\xrightarrow[O_2]{\text{dark}}$ violaxanthin
neoxanthin

c) Epoxidation in inhibited by N_2 whereas hydroxylation can take place. The inhibition is particularly striking for antheraxanthin ⟶ violaxanthin, in that the expoxidation was not been completely inhibited, a significant quantity of violaxanthin being formed in N_2 too. It is presumed that this is due to the oxygen remaining in the intercellular cavities of the leaf.

d) It is striking that in a normal atmosphere in the dark, the amount of neoxanthin increases, while in nitrogen it decreases considerably. It is presumed that, primarily under anaerobic conditions, the neoxanthin is converted violaxanthin.

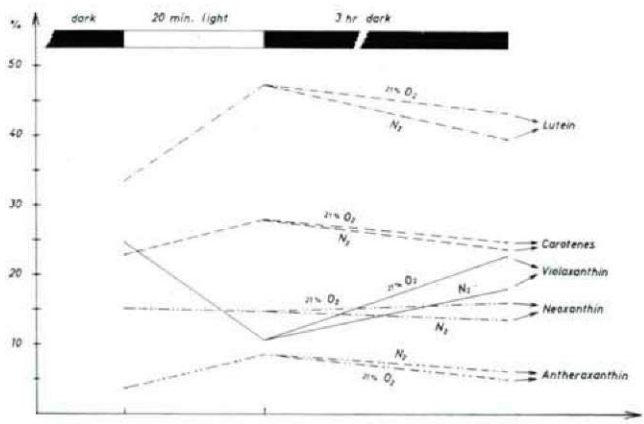

Fig. 5. The carotenoid changes taking place in a leaf of *Nonea lutea* as a result of dark- and light-induced reactions. (Explanation in the text.)

Summary

1. As a result of a strong illumination for a short time (5—40 minutes), the total carotenoid content in isolated leaves increases by 8—12%.

2. The effect of light is revealed under both aerobic and anaerobic conditions, primarily in desepoxidation reactions, but, in contrast to the literature data, zeaxanthin and lutein are not the end products of the transformation; they are in part dehydroxylated to give carotene. The new synthesis presumably plays a part in the 12—29% increase of the carotenes, in addition to the dehydroxylation.

3. The desepoxidation and the transformation of epoxy-carotenes into carotenes are strikingly increased by anaerobic conditions (pure N_2 gas).

4. The desepoxidation reactions are decreased by CO_2 under both aerobic and anaerobic conditions. A certain inhibitory effect exerted on desepoxidation is more expressed under anaerobic conditions.

5. As a result of the hydroxylation and epoxidation in the leaves previously illuminated and then kept in the dark, the amounts of carotenes and lutein decrease and that of violaxanthin increases. Under anaerobic conditions the amount of neoxanthin decreases; it presumably turns into violaxanthin.

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